(12) UK Patent Application (19) GB (11) 2 330 076 (13) A

(43) Date of A Publication 14.04.1999

(21) Application No 9821923.1

(22) Date of Filing 08.10.1998

(30) Priority Data
(31) 08949258 (32) 10.10.1997 (33) US

(71) Applicant(s)
S.S. Steiner, Inc.
(Incorporated in USA - New Jersey)
655 Madison Avenue, New York, NY 10021-8078,
United States of America

(72) Inventor(s)
Eric A. Johnson
Gerhard Julius Haas

(74) Agent and/or Address for Service
Sommerville & Rushton
45 Grosvenor Road, ST ALBANS, Herts., AL1 3AW,
United Kingdom

(51) INT CL[6]
A61K 35/78

(52) UK CL (Edition Q)
A5B BE B30X
U1S S2410

(56) Documents Cited
EP 0681029 A EP 0606599 A DE 002749274 A
US 5286506 A

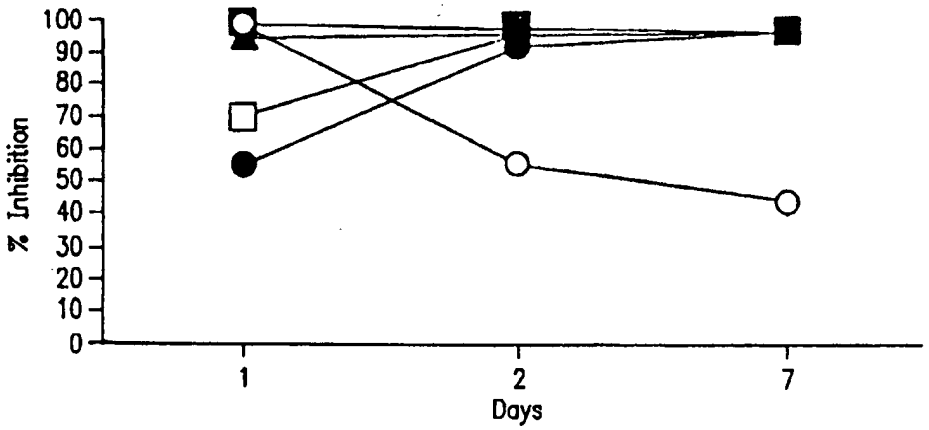
(58) Field of Search
UK CL (Edition Q) A5B BE
INT CL[6] A61K 35/78
ONLINE: EPODOC, JAPIO, PHARM, WPI

(54) Abstract Title
Use of hop extracts against botulism

(57) Hop extracts are useful as an antibacterial agent against the dangerous pathogens Clostridium botulinum and Clostridium difficile at levels below that at which a flavor from the acids contained therein is objectionable. More specifically, a process and associated product is described herein, comprising applying a solution of hop extract to a food, beverage or other medium so that the final concentration of hop ingredients is about 1 ppm or higher in order to inhibit the growth of Clostridium botulinum and/or Clostridium difficile.

GB 2 330 076 A

At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.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80
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60
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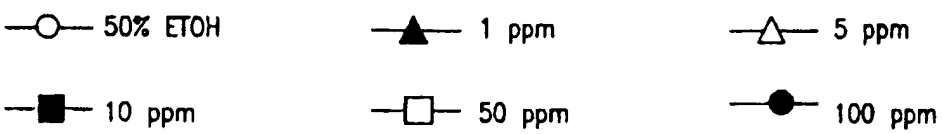
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. IA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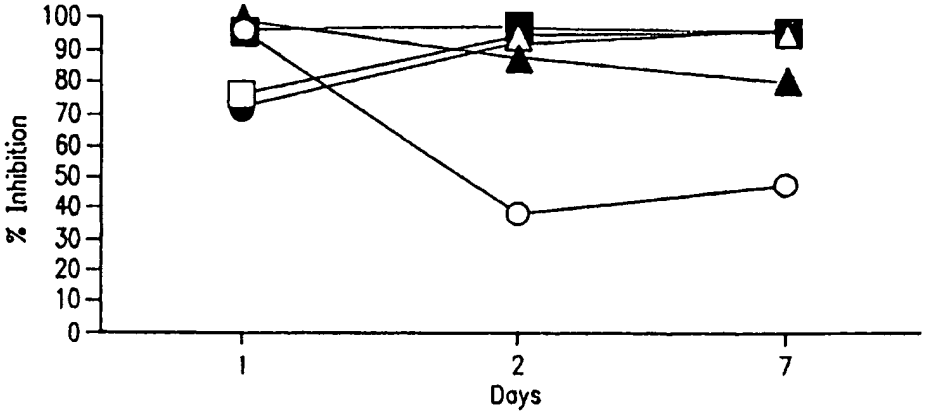
1 ppm
5 ppm
10 ppm
50 ppm
100 ppm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90
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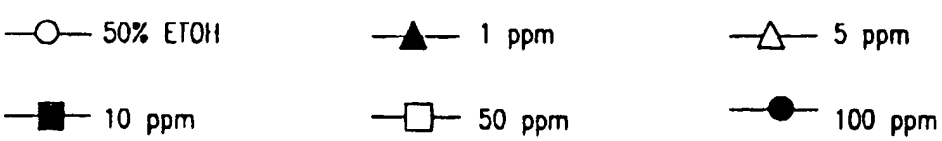
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. IB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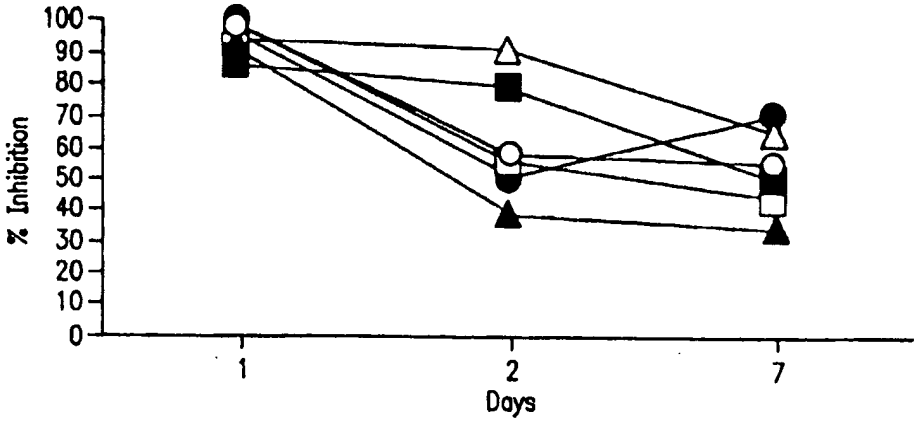
5 ppm
10 ppm
50 ppm
100 ppm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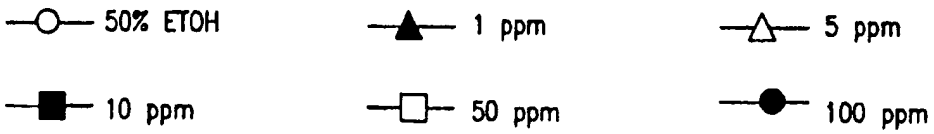
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. IC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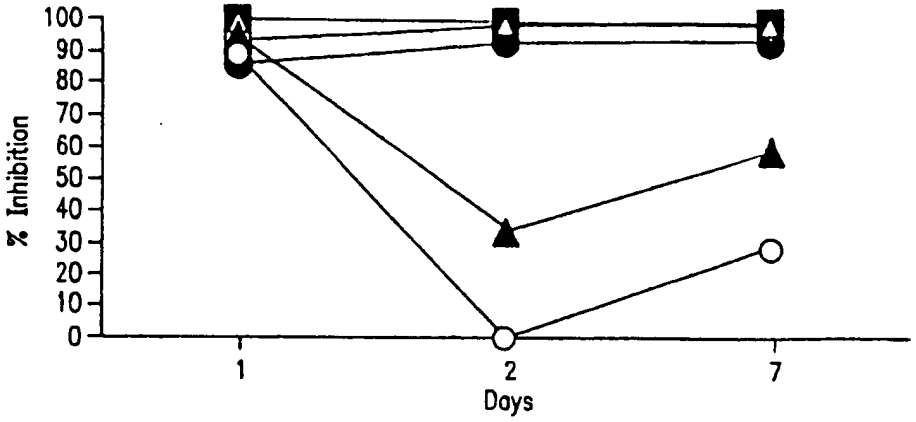
50 ppm
100 ppm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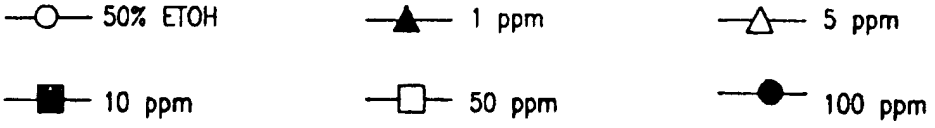
0
% Inhibition
1
2
7
Days

FIG. 2A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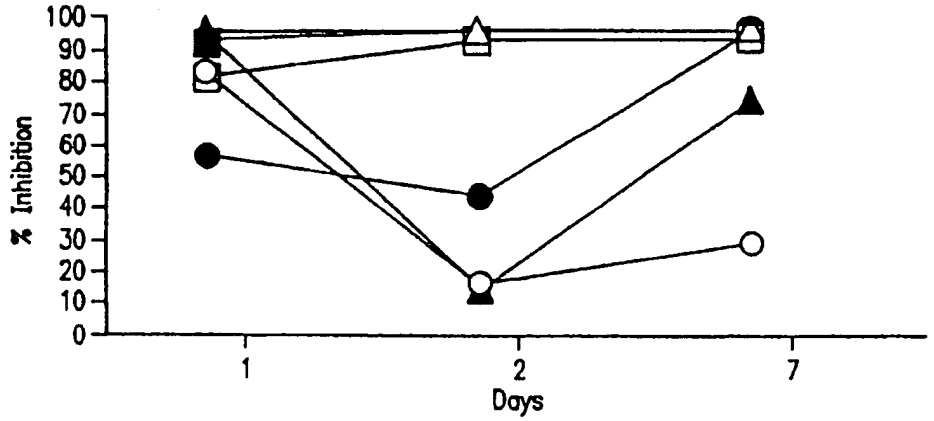
10 ppm
50 ppm
100 ppm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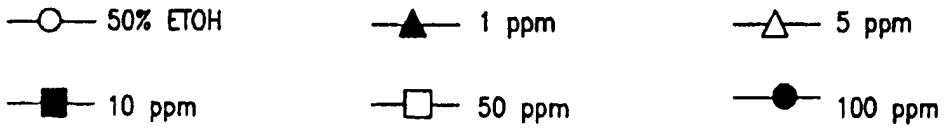
20
10
0
% Inhibition
1
2
7
Days

FIG. 2B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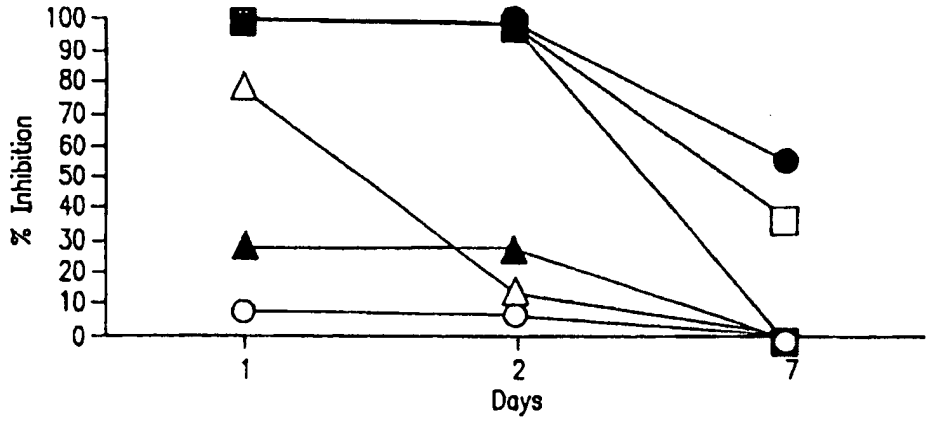
5 ppm
10 ppm
50 ppm
100 ppm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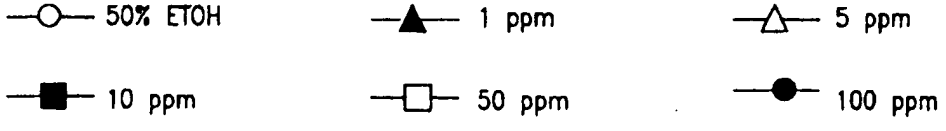
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 2C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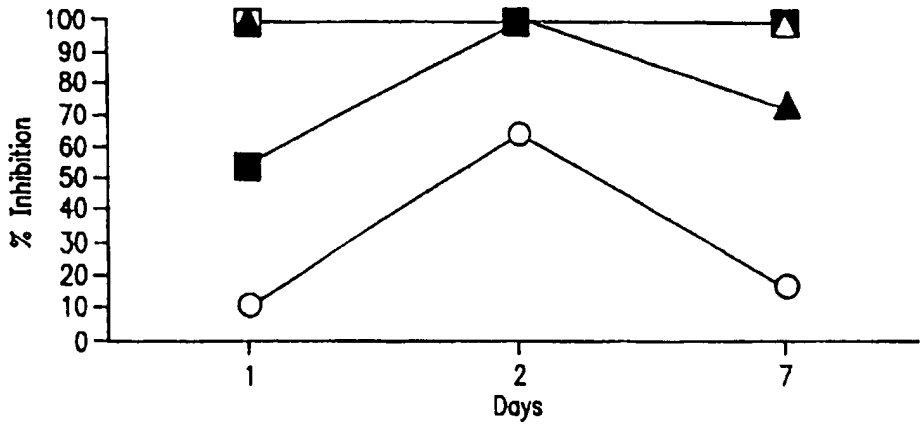
5 ppm
10 ppm
50 ppm
100 ppm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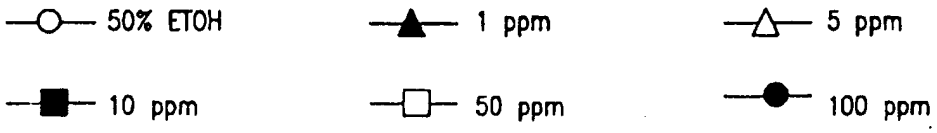
20
10
0
% Inhibition
1
2
7
Days

FIG. 3A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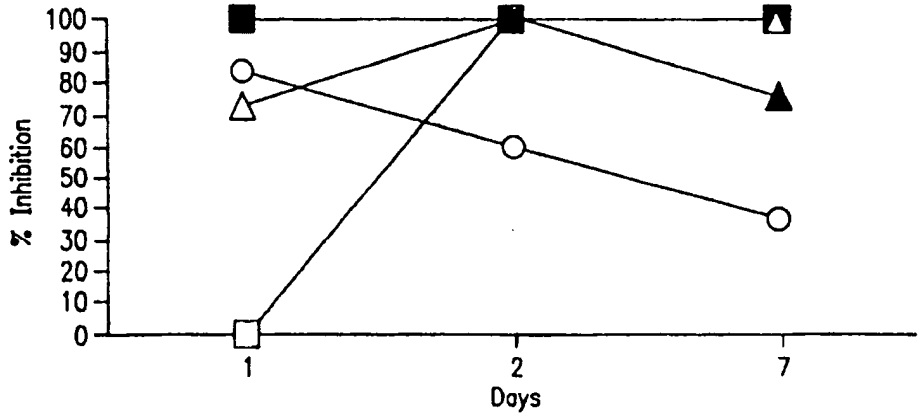
5 ppm
10 ppm
50 ppm
100 ppm


100
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40
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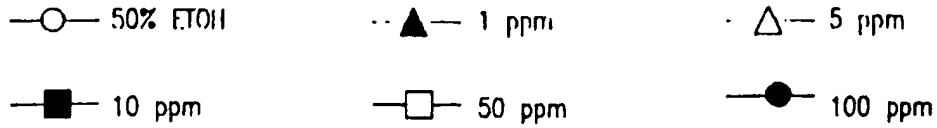
20
10
0
% Inhibition
1
2
7
Days

FIG. 3B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50 ppm
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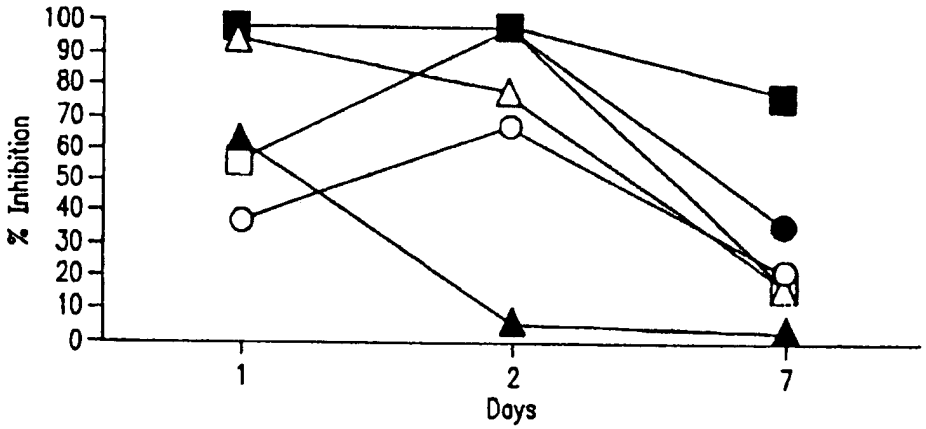
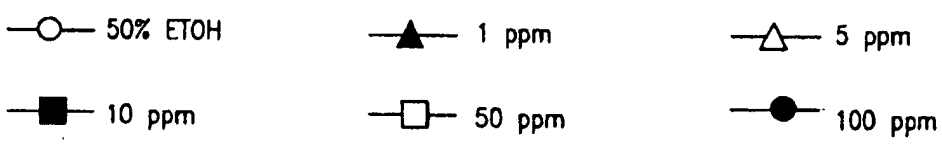

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 3C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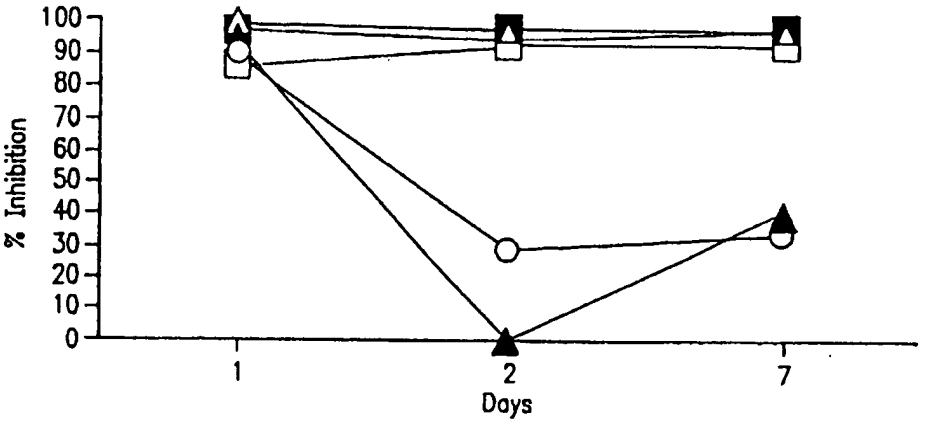
10 ppm
50 ppm
100 ppm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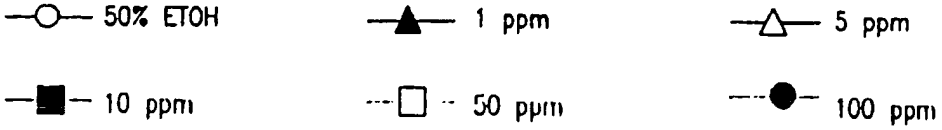
20
10
0
% Inhibition
1
2
7
Days

FIG. 4A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5 ppm
10 ppm
50 ppm
100 ppm

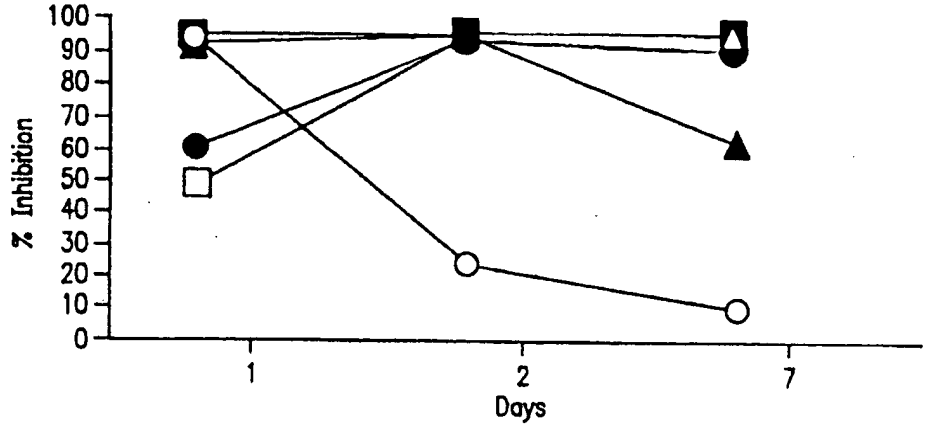

FIG. 4B

50% ETOH
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5 ppm
10 ppm
50 ppm
100 ppm

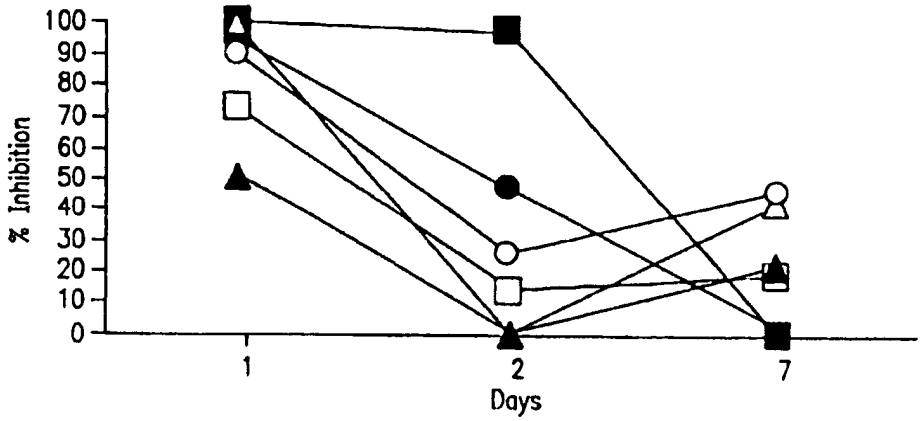

FIG. 4C

50% ETOH
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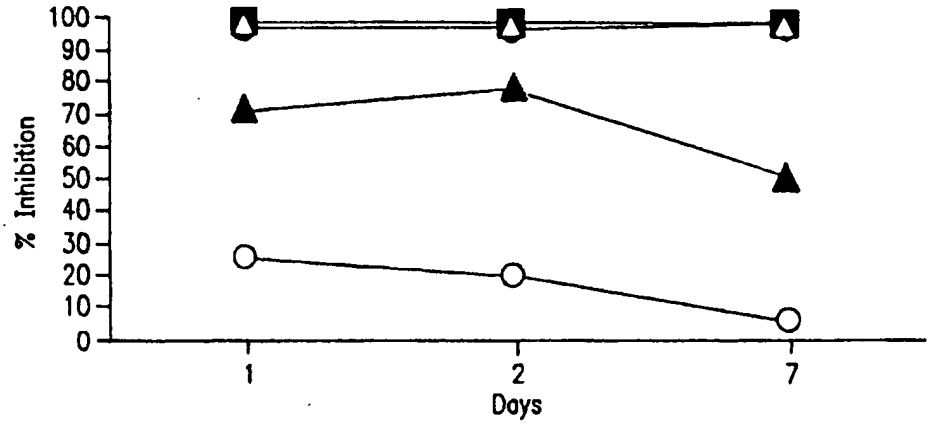
10 ppm
50 ppm
100 ppm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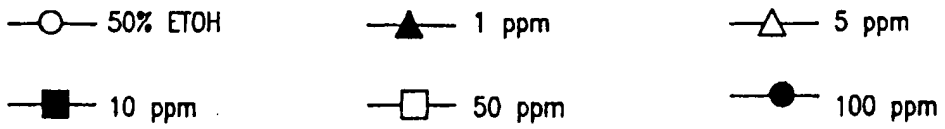
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 5A


50% ETOH
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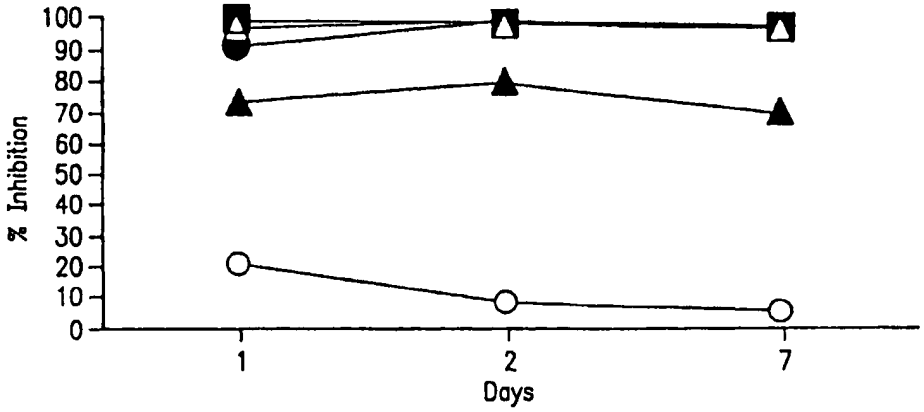
5 ppm
10 ppm
50 ppm
100 ppm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70
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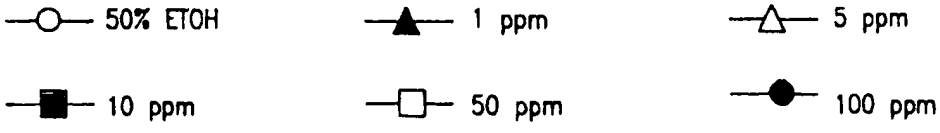
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 5B


50% ETOH
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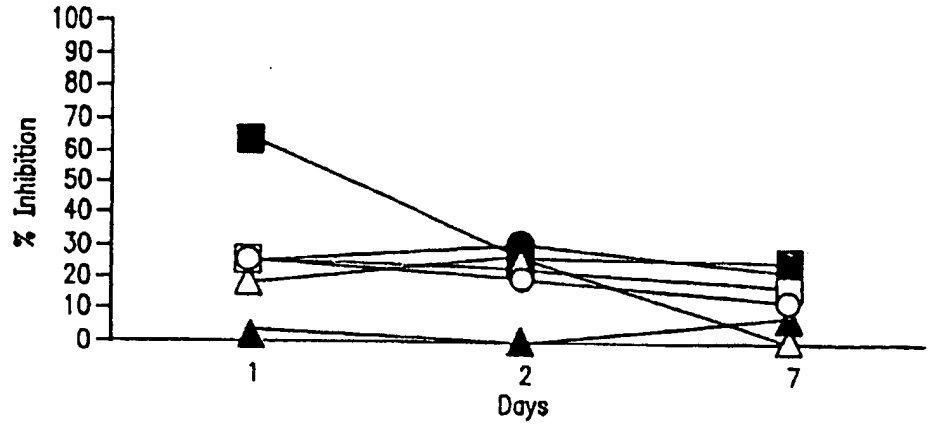
5 ppm
10 ppm
50 ppm
100 ppm


100
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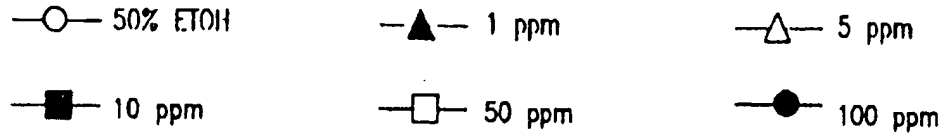
80
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 5C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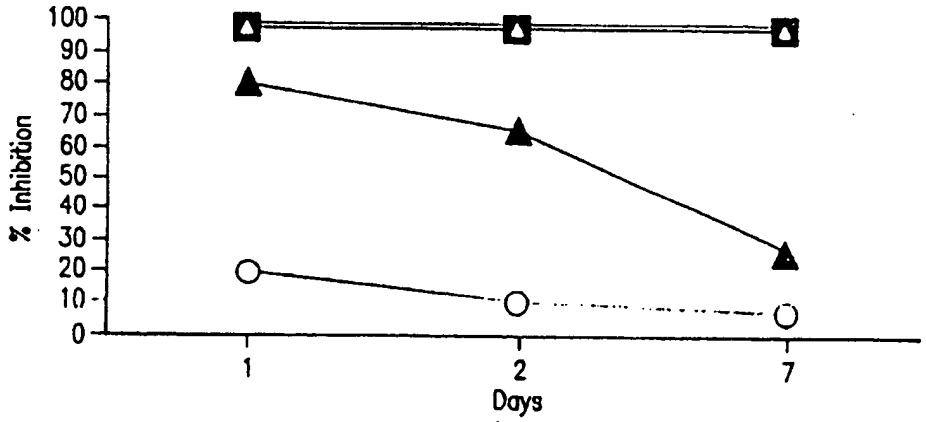
5 ppm
10 ppm
50 ppm
100 ppm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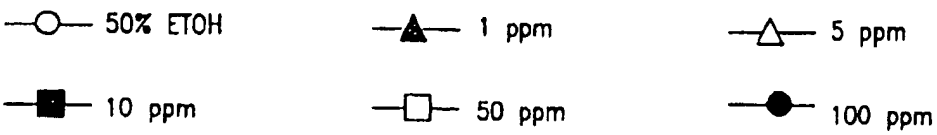
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 6A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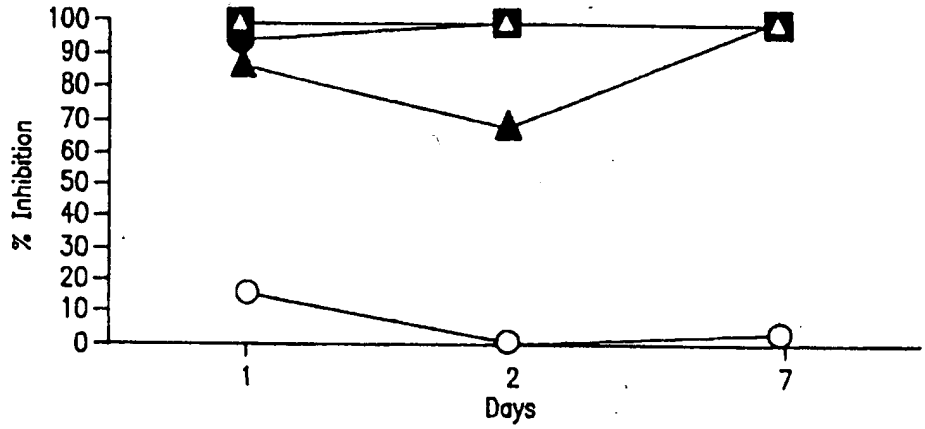
50 ppm
100 ppm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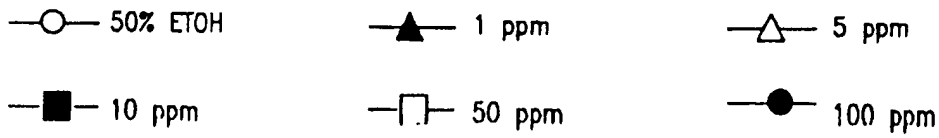
0
% Inhibition
1
2
7
Days

FIG. 6B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50 ppm
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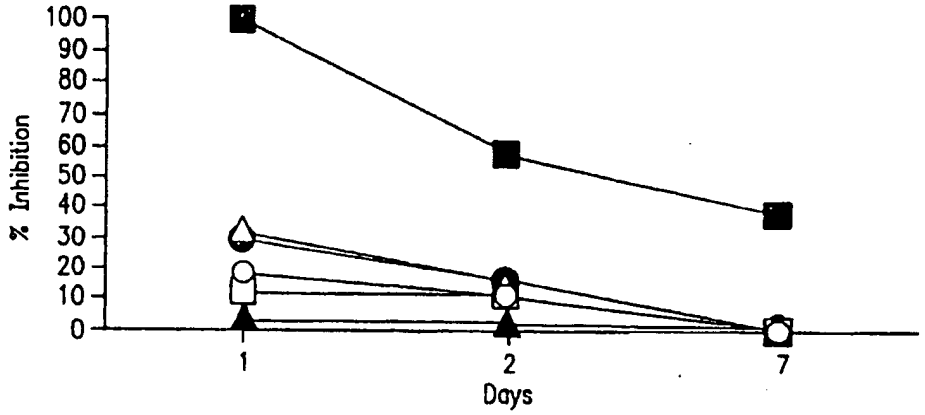
100
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80
70
60
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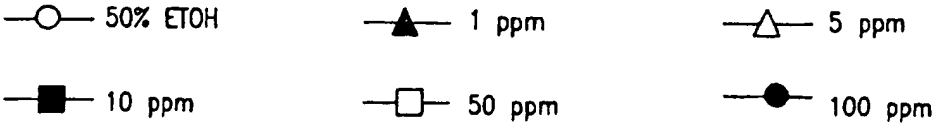
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 6C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10 ppm
50 ppm
100 ppm

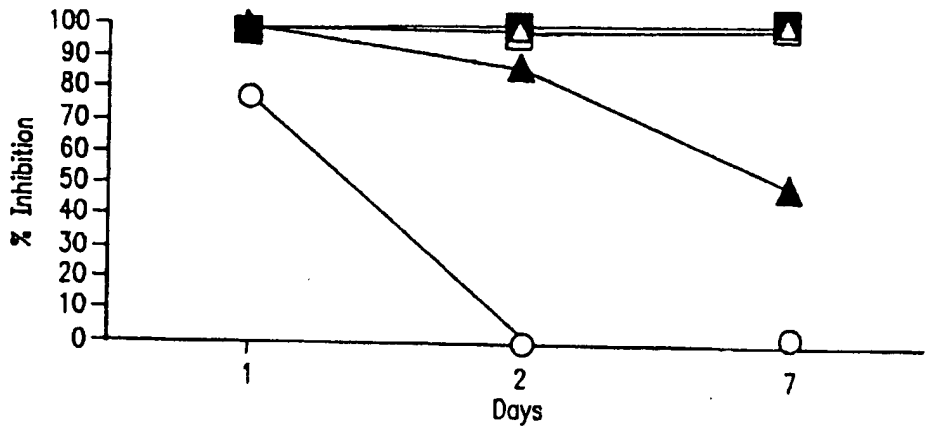
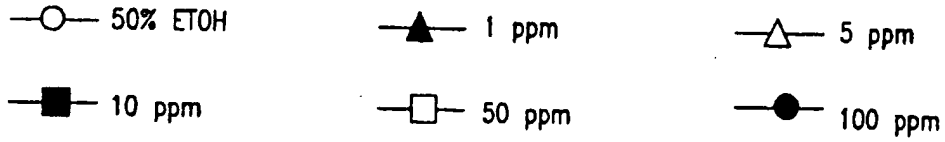

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 7A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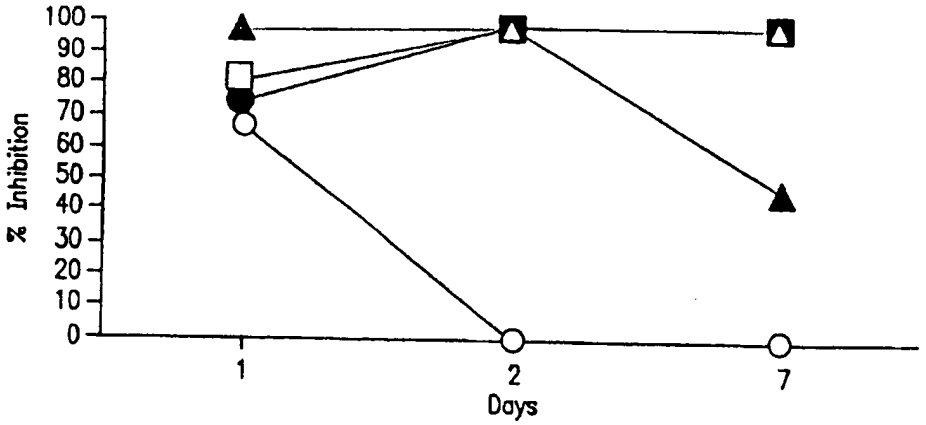
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40
30
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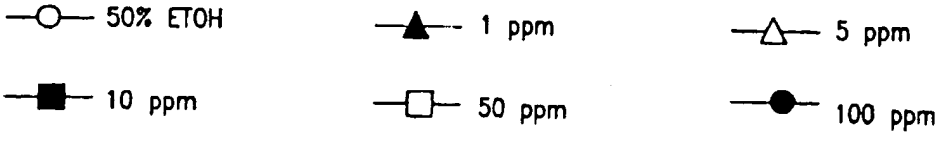
10
0
% Inhibition
1
2
7
Days

FIG. 7B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50 ppm
100 ppm

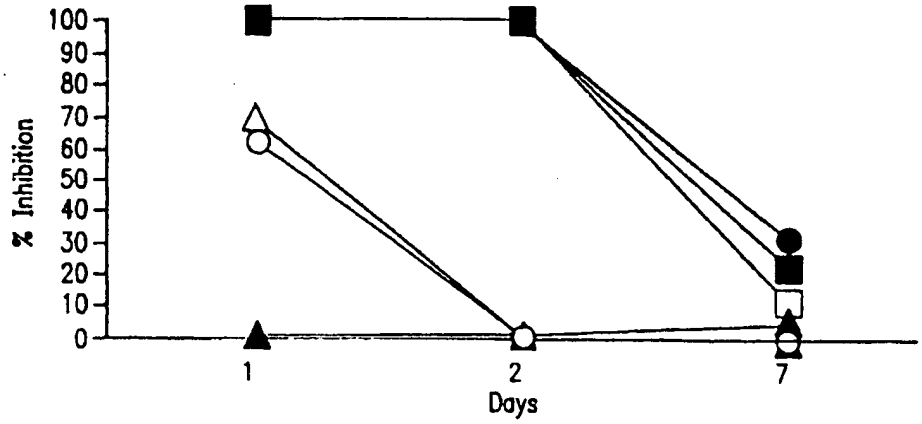
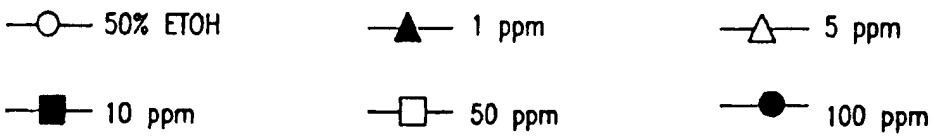

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 7C


50% ETOH
1 ppm
5 ppm
10 ppm
50 ppm
100 ppm

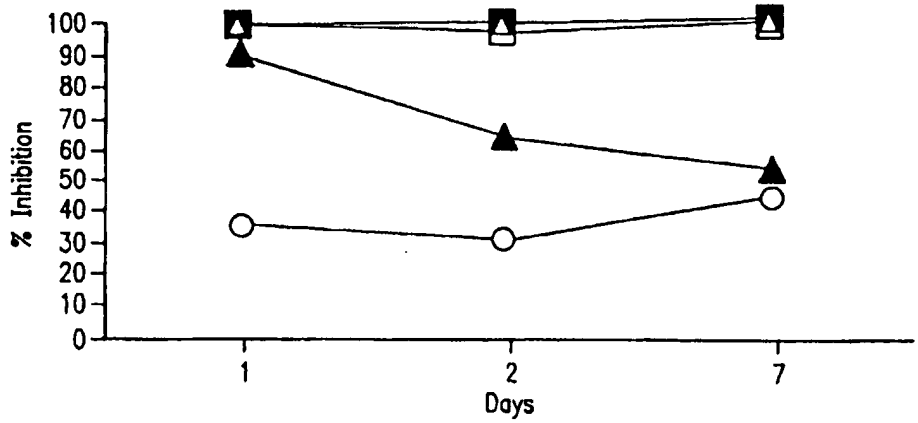
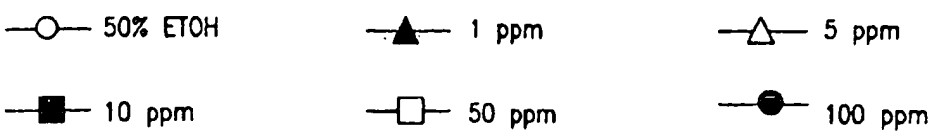

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 8A


50% ETOH
1 ppm
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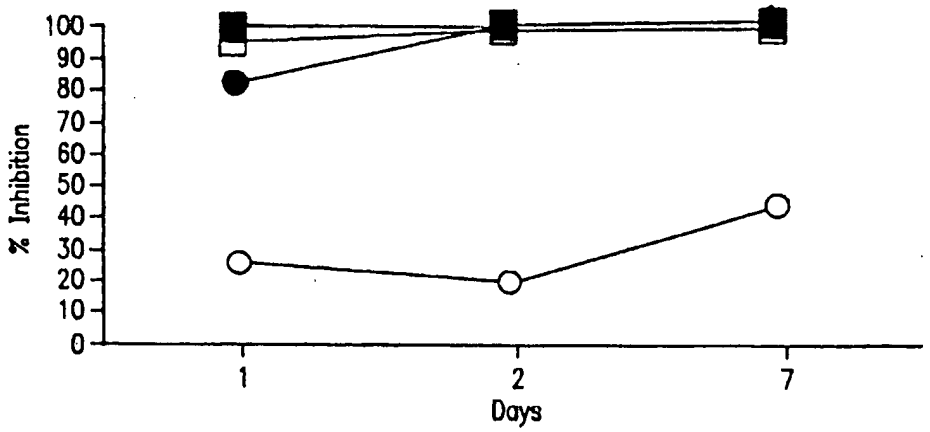
10 ppm
50 ppm
100 ppm


100
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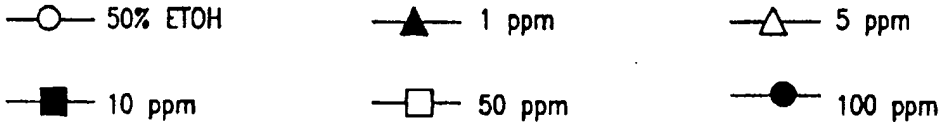
80
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 8B


50% ETOH
1 ppm
5 ppm
10 ppm
50 ppm
100 ppm

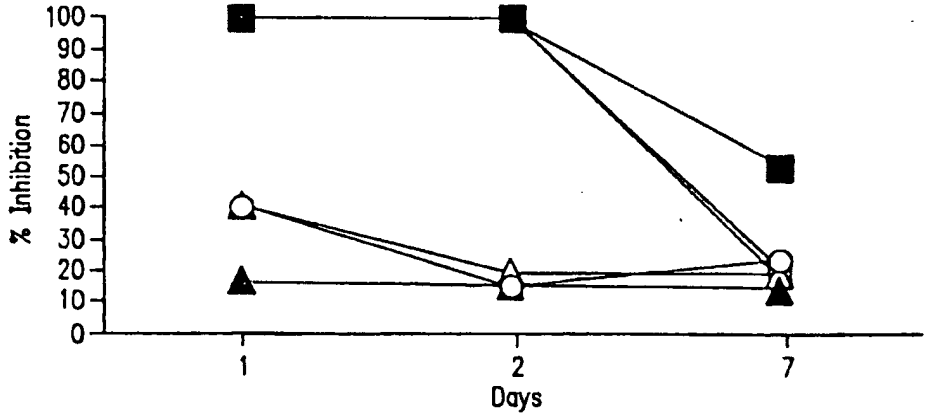
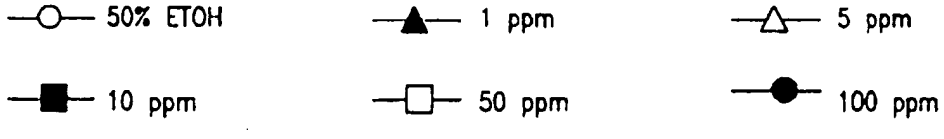

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 8C


50% ETOH
1 ppm
5 ppm
10 ppm
50 ppm
100 ppm

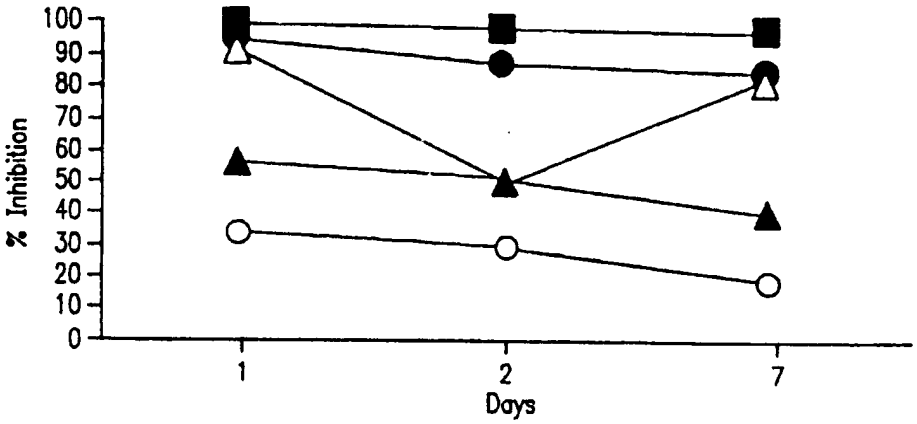
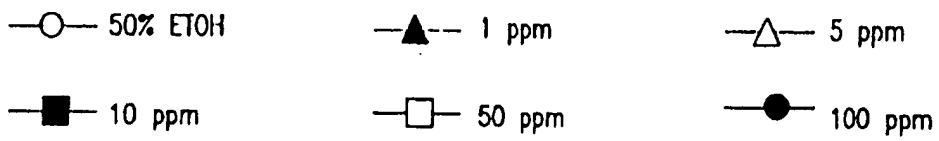

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 9A


50% ETOH
1 ppm
5 ppm
10 ppm
50 ppm
100 ppm

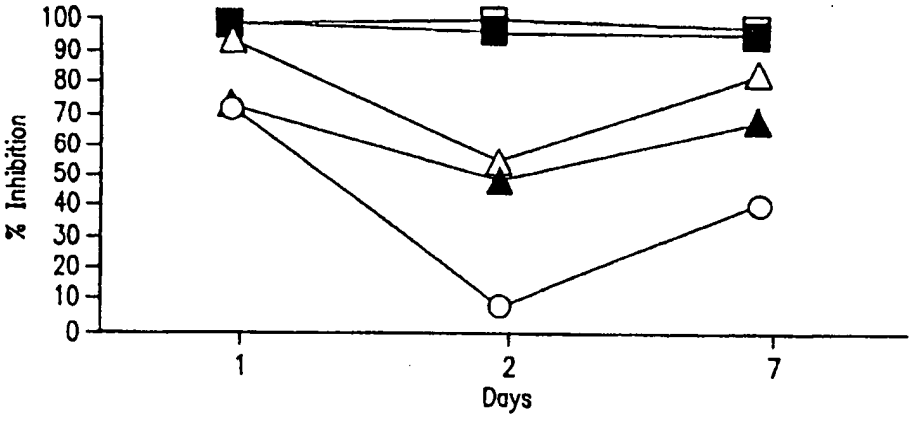
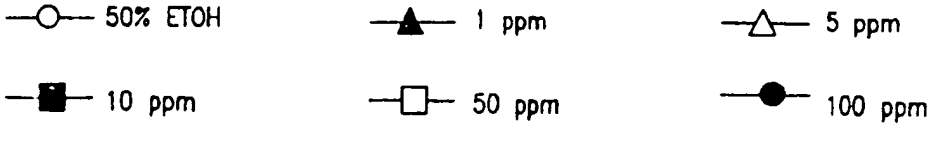

100
90
80
70
60
50
40
30
20
10
0
% Inhibition
1
2
7
Days

FIG. 9B


50% ETOH
1 ppm
5 ppm
10 ppm
50 ppm
100 ppm

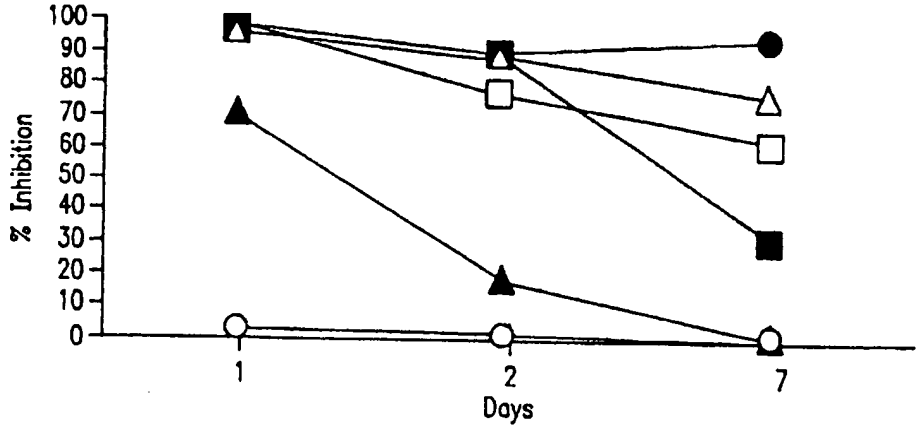
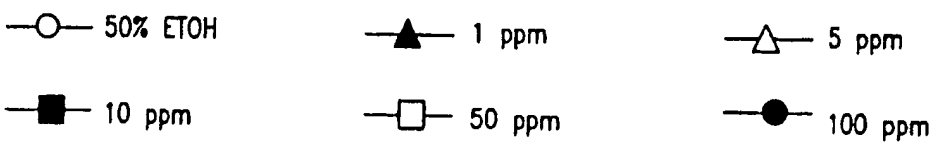


FIG. 9C



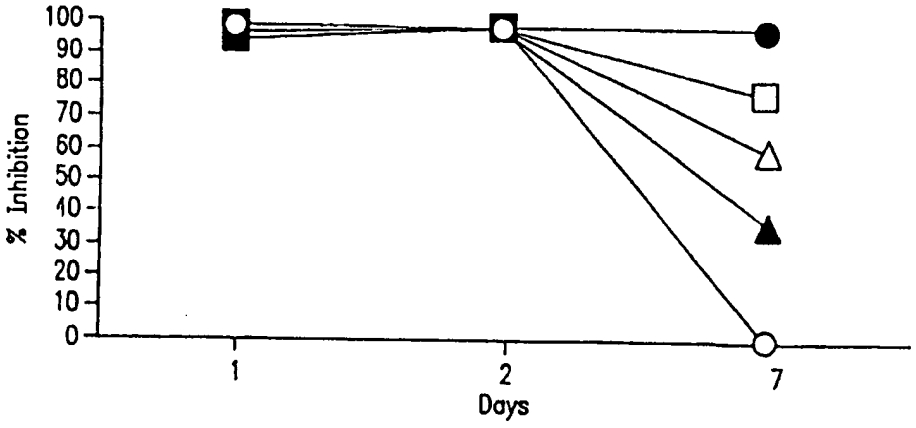
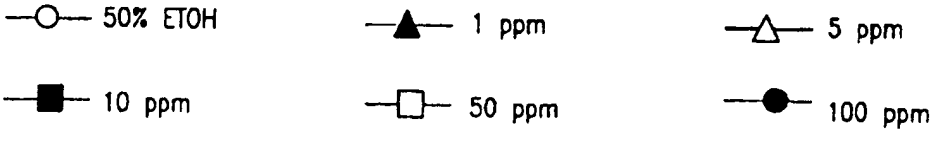


FIG. 10A



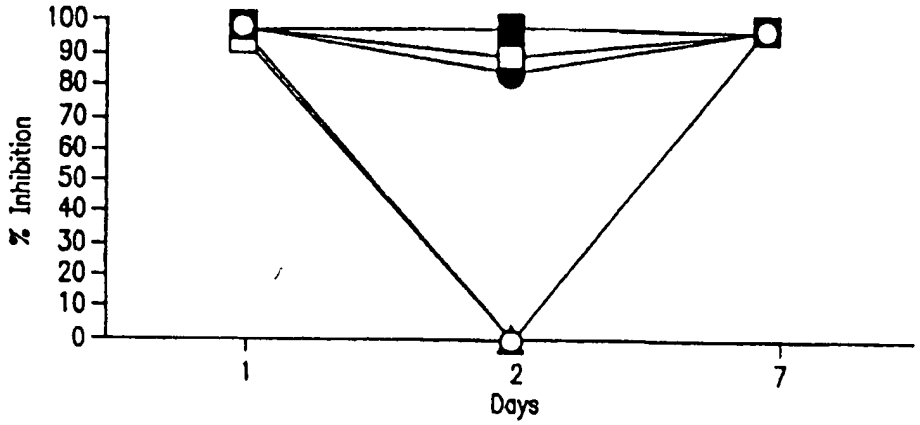
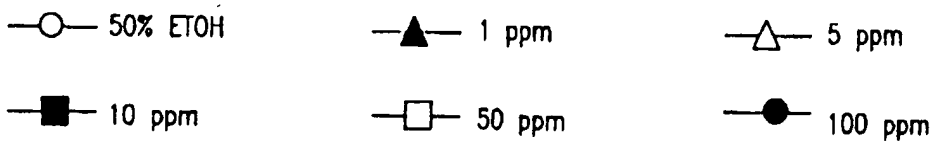


FIG. 10B



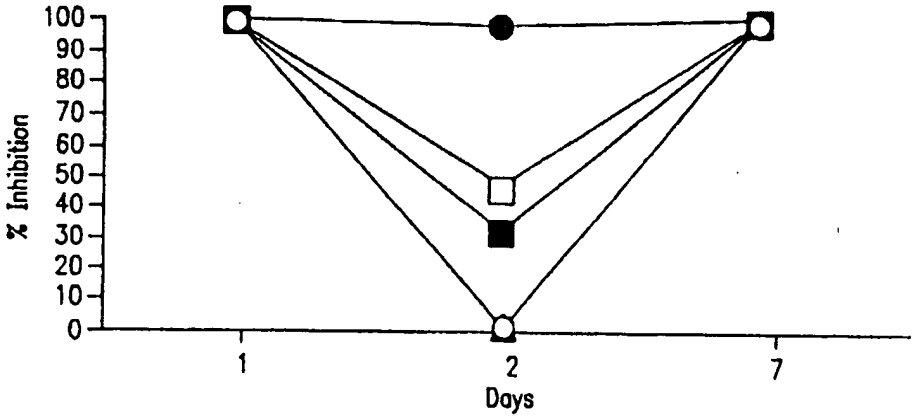
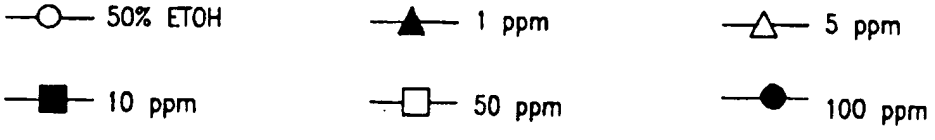


FIG. 10C



ANTIMICROBIAL ACTIVITY OF HOPS EXTRACT AGAINST CLOSTRIDIUM BOTULINUM AND CLOSTRIDIUM DIFFICILE

The present invention relates to the use of hop extracts for controlling Clostridium botulinum and Clostridium difficile.

The most prevalent groups of bitter acids found as components of hops are the alpha-acids and beta-acids, also referred to as humulones and lupulones, respectively. Both contribute bitterness to beer, but the alpha-acids are much more intense in this regard than the beta-acids. Producers of hop extracts have recently used liquid carbon dioxide under supercritical conditions. A by-product of the operation is a product which contains approximately 61 weight percent beta-acids, the remainder consisting essentially of other hop resins.

Quite apart from their use in beer, hops and hop acids have also been recognized as microbial inhibitors. More specifically, hop acids and resins have been shown to be primarily active against some gram positive bacteria and Mycobacteria. Activity against gram negative bacteria is far less pronounced. It has been suggested that the reduced effect was due to induced permeability of the cell membrane in gram positive bacteria, but was inactivated by the serum phosphatides in gram negative bacteria. Arch. Mikrobiol. 94, pp. 159-171, 1973.

Other more recent references have been identified, such as U.S. Patent No. 5,286,506 (1994) and Larson, Yu, Price, Haas and Johnson, International Journal of Food Microbiology, 1996, which report on the use of beta acids as extracted from hops for controlling Listeria. More specifically, 6 to 50 ppm of beta acids, as extracted from hops, was found in media to protect against Listeria monocytogenes contamination, while in foods, depending on the specific food, higher levels (100-300 ppm) were necessary.

Attention is also directed to the following references: Agricultural and Biological Chemistry, Vol. 49, No. 2, pp 399-404 (1985) which discloses that humulone, lupulone and related compounds were found to have antifungal activities; Dissertation Abstracts, Vol. 53-08B, 1991, pp. 3861, reports that compounds derived from hops possess antibacterial activity, and more specifically, the antibacterial

activity against Lactobacillus brevis was found to be pH-dependent; Journal of the Institute of Brewing, 99 (5) 405-411 (1993) reports on the results of studies investigating the ability of hop acids to inhibit beer spoilage activity; Journal of the Institute of Brewing, 99 (1) 43-48 (1993) reports on the antibacterial activity of hop bitter resins derived from recovered hopped worts. More specifically, strains of thermophilic Bacillus spp were identified which failed to grow in certain sweet worts derived from mashes to which centrate (recovered hop wort) had been added; J. Food Prot. Vol 57, No. 1, pp 59-61 (1994) reports on the antimicrobial activity of hop resins against Streptococcus salivarius. The two hop resins used were iso-alpha acid and beta resin; Agric. Biol. Chem., Vol. 49, No. 2, pp. 399-403 (1985) discloses that humulone, lupulone and related compounds as having antifungal activities; Lebensm. Ind. Vol. 28, No. 7, pp. 311-315 (1981) reports that tests showed that hop extract and isomerized hop extract have similar anti-microbial properties like hops, but the antimicrobial effect of the hops in beer production was low. J. Appl. Bacteriol., Vol. 72, No. 4, pp. 327-324 (1992) considered the antibacterial effect of weak acids derived from the hop plant Humulus lupulus. The antibacterial activity of trans-isohumulone was about 20 times greater than that of humulone, 11 times greater than colupulone, and nine times greater than that of trans-humulinic acid when the degree of ionization was taken into account.

However not all gram positive bacteria are sensitive to hop resins as is well known to the Brewer and see J. Fernandez and Will Simpson in J. App Bacteriology, 75 315-319 (1993). Also G. Haas and B. Barsoumian in J. Food Protection 57, 59-61 (1994) worked with a strain of Bacillus subtilis which was resistant.

None of the art noted above deals with the control of botulism, which is well-known as an acute intoxication manifested by neuromuscular disturbances after ingesting food containing a toxin elaborated by Clostridium botulinum. The causative agent is actually one of several types of exotoxins elaborated by the sporulating, anaerobic bacillus Clostridium botulinum, which causes human poisoning. Botulinum toxins are highly poisonous proteins resistant to digestion by gastrointestinal enzymes. Clostridium difficile is one of the major causes of diarrheal disease

particularly in elderly humans treated with antibiotics. Very few antibiotics are effective and treatment of this infection is difficult at best. Only vancomycin of the well known antibiotics seems to be useful in treatment. Helicobacter pylori is a common cause of gastric ulcers and chronic active gastritis in humans. Ulcer relapses are common in humans treated with antibiotics or bismith nitrate. Other intervention strategies have to be sought and a nutritional or dietetic approach would be highly desirable.

The present invention relates to the discovery that hops extract or the components of hops extract are useful as an antibacterial agent against dangerous pathogens Clostridium botulinum and Clostridium difficile. More specifically, a process and associated product is described herein, comprising applying at least about 1 ppm or greater, by weight, of beta acids, or hop extracts to inhibit growth of Clostridium botulinum and Clostridium difficile. Medications, disinfectant solutions or pharmaceutical compositions containing these materials may also be used.

Figs. 1A, 1B and 1C illustrate the inhibition of Clostridium botulinum 56A by hop extracts "a" (41% beta, 12% alpha and 47% desoxy alpha, hop oils and hop waxes), "b" (65% w/v beta acids) and "c" (6% w/v post beta-acids in Tween 80), at different concentrations in ethanol (50%) solution.

Figs. 2A, 2B and 2C illustrate the inhibition of Clostridium botulinum 62A by hop extracts a, b and c, as described above.

Figs 3A, 3B and 3C illustrate the inhibition of Clostridium botulinum 213B by hop extracts a, b and c, as described above.

Figs. 4A, 4B and 4C illustrate the inhibition of Clostridium botulinum Lamanna-Okra B by hop extracts a, b and c, as described above.

Figs 5A, 5B and 5C illustrate the inhibition of Clostridium botulinum Alaskan E by hop extracts a, b and c, as described above.

Figs 6A, 6B and 6C illustrate the inhibition of Clostridium botulinum Beluga E by hop extracts a, b and c, as described above.

Figs 7A, 7B and 7C illustrate the inhibition of Clostridium botulinum 17 by hop extracts a, b and c, as described above.

Figs 8A, 8B and 8C illustrate the inhibition of Clostridium botulinum 4848B by hop extracts a, b and c, as described above.

Figs 9A, 9B and 9C illustrate the inhibition of Clostridium difficile 43255 by hop extracts a, b and c, as described above.

Figs 10A, 10B and 10C illustrate the inhibition of Clostridium difficile 10463 by hop extracts a, b and c, as described above.

The present invention relates to the discovery that hop extracts or fractions are useful as a preservative inhibiting the pathogens Clostridium botulinum and Clostridium difficile as agents to prevent illness caused by said pathogens. Three different hop extracts were evaluated to demonstrate the broad applicability of the present invention.

The hop extracts as used herein may comprise solvent extracted hops, or liquid CO_2 or supercritical CO_2 gas extracted hops. Particularly preferred are CO_2 liquid or CO_2 critical gas extracts. Generally, the hop extracts are added to a food product or other vehicle, in solution, to achieve at least about one part per million, by weight, of beta acids in the GI tract or stomach. Amounts less than about 1 ppm, by weight, beta acids, does not appear to provide protection against Clostridium botulinum and Clostridium difficile. The solution preferably contains about 5 ppm - 100 ppm, by weight, of beta acids. The upper level is dictated by taste and solubility.

Figs. 1-10 collectively illustrate the experimental results confirming the antimicrobial effects disclosed herein. More specifically, and as noted above in the brief description of the drawings, Figs. 1A through 10A reference the use of hop extract "a", which contained 41 % beta, 12 % alpha and the remaining 47% contained a mixture of desoxy-alpha, hop oils and hop waxes. Figs. 1B-10B refers to the use of hop extract "b", which contained 65% (w/v) beta acids. Fig. 1C-10C refer to hop extract "c" which contained 6% (w/v) post beta acids in Tween 80. In each case the hop extract was made up as a solution in 50 % ethanol, and added to achieve 1, 5, 10, 50 and 100 ppm. A control with 50 % ethanol but without hop resin was included.

The organisms targeted in this invention included 8 strains of Clostridium botulinum and two strains of Clostridium difficile, as listed below:

Clostridium botulinum:

- Proteolytic type A: 56A, 62A
- Proteolytic type B: 213B, Lamanna-Okra B,
- Non-proteolytic type B: 17B, 4848B,
- Non-proteolytic type E: Alaska E, Beluga E

Clostridium difficile:

- 43255
- 10463

These organisms are toxicogenic and have been involved in human intoxication or infections. The inhibition of Clostridium botulinum by hop extracts in broth media was established as follows:

Eight strains of Clostridium botulinum were inoculated as spores separately into tubes of 10 ml trypticase peptone-glucose-yeast extract (TPGY) broth containing 5 different levels (1, 5, 10, 50 and 100 ppm) of three hop extracts. Before inoculation, spores were treated with a heat treatment to activate them in order to achieve maxiumum germination. For proteolytic strains, spores were heat treated at 80°C for 10 min and spores from non-proteolytic strains were treated at 60°C for 20 min. Dilutions were made to have an initial inoculum ranging between $2x10^3$ and $3X10^3$ spores/ml.

Clostridium difficile strains were incubated in Brain Heart Infusion (BHI), 0.1% Yeast Extract (UYE) broth at 37°C.

As noted, hop extracts "a", "b" and "c" were tested at five different concentrations in the final medium: 1, 5, 10, 50, and 100 ppm. The tubes were incubated at 30°C for one week. Growth (measured as increased absorbance) was monitored by optical density (O.D. at 660 nm) at one, two and seven days. Controls (only broth) and ethanol controls were inoculated with the spores but hop extracts were not added. All combinations of variables were tested in duplicate and replicated at least once.

With attention now directed at Figs. 1A, 1B and 1C through 10A, 10B and 10C, as illustrated therein, hop extracts "a" and "b" produced inhibitory activity towards all eight Clostridium botulinum strains at a concentration as low as 1 ppm, and more preferably at concentrations of 5, 10, 50 and 100 ppm. Accordingly, 5-100 ppm of hop extracts "a" and "b" were found as the most preferred in the broad context of the present invention as applied to the Clostridium botulinum strain. Similarly, spores of Clostridium difficile strains were inhibited by hop extracts "a", "b" and "c" also at concentrations as low as 1 ppm, and more preferably at concentrations of 5, 10, 50 and 100 ppm, establishing effectiveness at the similar preferred range of 5-100 ppm.

The results above confirm that with regards to botulinum, hop extracts, quite apart from the known use in beer, have proven to be uniquely suited to provide effective inhibitory activity against this very important food pathogen. In addition, hop extracts also have shown their inhibitory activity against Clostridium difficile strains. The hop extracts therefore may be conveniently incorporated into a food product by dipping or spraying the food product with a solution of the extracts or alternatively added to a suitable vehicle such as an oral formulation to treat or prevent disease caused by the above microbes.

In addition to the above, those skilled in the art will recognize herein that the present invention also relates to the preparation of disinfectant compositions to inhibit growth, and pharmaceutical compositions to prevent transmission, of the pathogens identified herein, wherein said compositions comprise at least 1 ppm of hop extracts, or more preferably, 5, 10, 50 and 100 ppm, and/or the specific range between about 5-100 ppm.

CLAIMS

1. A process comprising applying hop extract or the components of hop extract to a food product or beverage to inhibit growth of Clostridium botulinum in said food product or beverage, wherein to incorporate about 1 ppm or greater of said hop extract or said components of hop extract.

2. The process of claim 1, characterized by one or more of the following features:

(a) wherein said hop extract contains a beta-acid;

(b) wherein the extract is added as a solution in ethanol to achieve at least 5 ppm concentration;

(c) wherein the extract is solubilized by Tween 80 or other surface active agents;

(d) wherein said food product or beverage contains 5-100 ppm hop extract.;

(e) wherein the hop extract contains a mixture of beta-acid, alpha-acid and desoxy alpha acid, along with hop oils, hop waxes and/or other hop constituents;

(f) wherein the hop extract contains about 65% beta acids;

(g) wherein the solution of hop extracts is applied to the food product by dipping the food product in said solution of hop extracts; and

(h) wherein the solution of hop extracts is applied to the food product by spraying said solution onto said food product.

3. A solid food containing about 1 ppm or greater amount of hop extracts to prevent growth of Clostridium botulinum.

4. The food product of claim 3, characterized by one or more of the following features:

(a) which contains 5-100 ppm hop extract;

(b) wherein said hop extracts contain a mixture of beta-acids, alpha-acids, desoxy-alpha acids, hop oils, hop waxes and/or other hop constituents; and

(c) wherein said hop extracts contain beta acids.

5. A process comprising applying a solution containing hop extract to a food product or beverage to inhibit growth of Clostridium difficile in the intestine or stomach, wherein said product or beverage provides at least 1 ppm of said hop extract to said intestine or stomach.

6. The process of claim 5, characterized by one or more of the following features:

(a) wherein said hop extract contains a mixture of beta-acids, alpha-acids, desoxy-alpha acids, hop oils and hop waxes;

(b) wherein said hop extract is added in ethanolic solution;

(c) wherein the hop extract contains beta acids;

(d) wherein the hop extract is solubilized by Tween 80 or other surface active agents;

(e) wherein the solution of hop extract is applied to the food product by dipping the food product in said solution of hop extract; and

(f) wherein the solution of hop extract is applied to the food product by spraying said solution onto said food product.

7. A food product or beverage comprising hop extract or the components of hop extract wherein said food product or beverage delivers about 1 ppm or greater of hop extract or the components of hop extract in the stomach or intestine to prevent growth of Clostridium difficile.

8. The food product of claim 7, characterized by one or more of the following features:

(a) wherein the hop extract has been added to said food as an ethanolic solution;

(b) wherein said food contains 5-100 ppm hop extract;

(c) wherein said hop extract contains a mixture of beta-acids, alpha-acids, desoxy-alpha acids, hop oils, hop waxes and/or other hop constituents; and

(d) wherein said hop extracts contains 65 % (w/v) beta acids.

9. A disinfectant composition to prevent transmission of Clostridium botulinum and/or Clostridium difficile comprising about 1 ppm or greater amount of hop extract or components of hop extract.

10. A pharmaceutical composition to inhibit the growth of Clostridium difficile and/or Clostridium botulinum comprising about 1 ppm or greater amount of hop extract or components of hop extract.

Patent Office

INVESTOR IN PEOPLE

Application No: GB 9821923.1 **Examiner:** Diane Davies
Claims searched: 7-10 **Date of search:** 18 January 1999

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.Q): A5B: BE

Int Cl (Ed.6): A61K 35/58

Other: Online: EPODOC, JAPIO, PHARM, TXTE, WPI

Documents considered to be relevant:

Category	Identity of document and relevant passage		Relevant to claims
X	EP 0606599 A	(Miller Brewing Co.) Whole document: oral care compositions containing hop acids or their salts to inhibit bacterial growth.	7-10
X	EP 0681029 A	(Zuckerforschung Tulln GmbH) Whole document: preventing the growth of thermophilic microorganisms in sugar solutions by using hop extracts.	7-10
X	US 5286506 A	(Bio-Technical Resources) Whole document: inhibition of food pathogens such as *Clostridium perfringes* by using hop extracts.	7-10
X	DE 2749274 A	(S.S. Steiner Inc.) Whole document: use of hop extracts as a bacteriacide in deodorant compositions.	7-10

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.